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Stochastic Transmission of Multiple Genotypically Distinct *Anaplasma marginale* Strains in a Herd with High Prevalence of *Anaplasma* Infection

Guy H. Palmer,[1]* Donald P. Knowles, Jr.,[2] Jose-Luis Rodriguez,[1] David P. Gnad,[3] Larry C. Hollis,[4] Twig Marston,[4] and Kelly A. Brayton[1]

Program in Vector-borne Diseases, Washington State University,[1] and Animal Diseases Research Unit, U.S. Department of Agriculture-Agricultural Research Service,[2] Pullman, Washington, and Department of Clinical Sciences[3] and Department of Animal Sciences and Industry,[4] Kansas State University, Manhattan, Kansas

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Multiple genotypically unique strains of the tick-borne pathogen *Anaplasma marginale* occur and are transmitted within regions where the organism is endemic. In this study, we tested the hypothesis that specific *A. marginale* strains are preferentially transmitted. The study herd of cattle ($n = 261$) had an infection prevalence of 29% as determined by competitive inhibition enzyme-linked immunosorbent assay and PCR, with complete concordance between results of the two assays. Genotyping revealed the presence of 11 unique strains within the herd. Although the majority of the individuals (70 of 75) were infected with only a single *A. marginale* strain, five animals each carried two strains with markedly distinct genotypes, indicating that superinfection does occur with distinct *A. marginale* strains, as has been reported with *A. marginale* and *A. marginale* subsp. *centrale* strains. Identification of strains in animals born into and infected within the herd during the period from 1998 to 2003 revealed no significant difference from the overall strain prevalence in the herd, results that do not support the occurrence of preferential strain transmission within a population of persistently infected animals and are most consistent with pathogen strain transmission being stochastic.

Anaplasma marginale is the most globally prevalent tick-transmitted pathogen of livestock, with regions of endemicity on all six permanently populated continents (13). In North America, *A. marginale* exhibits dramatic strain diversity, with numerous distinct genotypes having been identified in the past 15 years (1, 5, 6, 8, 14). As *A. marginale* is not passed transovarially in the tick and thus cannot be maintained between generations, transmission requires the presence of infected mammalian reservoir hosts (12, 17). Consequently, the presence of multiple distinct strains within the population of infected reservoir hosts provides potential diversity for transmission. A previous study in a region of eastern Oregon where the organism is endemic identified six closely genetically related *A. marginale* strains within a herd of persistently infected cattle, an observation consistent with ongoing transmission of multiple strains within the herd (14). Independent transmission of multiple strains within a herd is supported by the isolation of distinct *A. marginale* strains, each from an individual cow with acute anaplasmosis, within a period of several months (7). It is unknown whether transmission of a specific *A. marginale* strain is stochastic, merely reflecting the strain composition present in the herd, or whether there is preferential transmission of specific strains. The identification of both qualitative (transmissible or not by a specific tick vector) and quantitative (strain-specific differences in the number of organisms per infected tick) differences in transmissibility among *A. marginale* strains provides a basis for preferential transmission (2, 9, 16, 19).

* Corresponding author. Mailing address: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-6033. Fax: (509) 335-8529. E-mail: gpalmer@vetmed.wsu.edu.

To test the hypothesis that specific *A. marginale* strains are preferentially transmitted within a herd with a high prevalence of infection, we analyzed the strain composition in 75 animals born from 1990 to 1999 and subsequently infected with *A. marginale* within the herd. The study herd was located at Kansas State University and contained 261 cows selected for initial screening. All of the cows in the present study were born into this herd and remained within the herd for the duration of the study; all blood samples used for determination of prevalence and identification of strain genotype were collected in early spring prior to the transmission season. Initially, 75 animals were identified as infected with *A. marginale* with the MSP5 competitive inhibition enzyme-linked immunosorbent assay (CI-ELISA) (11, 18). All 75 seropositive animals were subsequently shown to be *A. marginale* PCR positive by using *msp5*- or *msp1*α-specific primers with genomic DNA extracted from whole blood collected in heparin, procedures previously described in detail (9, 14, 18). Thus, *A. marginale* prevalence within the herd was 29%, and there was 100% concordance between MSP5 CI-ELISA serology and the PCR assays. The *A. marginale* genotype was determined by sequencing the *msp1*α gene and identifying the number and sequence of the 84- or 87-bp repeats in the 5′ region of the gene. Briefly, primers in the conserved regions flanking the strain-specific repeat region of *msp1*α (forward, 5′-GTGCTTATGGCAGACATTTCC-3′; reverse, 5′-CTCAACACTCGCAACCTTGG-3′) were used in PCR amplification as previously described (14). Amplified fragments were identified by agarose gel electrophoresis and cloned into PCR-4 TOPO vector using the TOPO-TA cloning kit (Invitrogen), and TOP10 *Escherichia coli* competent cells were transformed. If more than a single amplicon was detected, the amplicons were excised and cloned individually.

TABLE 1. Distribution of *A. marginale msp1*α genotypes within the herd

Animal	ELISA	PCR	*msp1*α genotype	Animal	ELISA	PCR	*msp1*α genotype
3261	+	+	BB	3201	+	+	EMϕ
8082	+	+	BB	4084	+	+	EMϕ
4102	+	+	BBB	4309	+	+	EMϕ
2267	+	+	BBBB	3293	+	+	EMϕ
7304	+	+	BBBB	4503	+	+	EMϕ
8405	+	+	BBBB	5077	+	+	EMϕ
0141	+	+	BBBBB	5086	+	+	EMϕ
4087	+	+	BBBBB	5306	+	+	EMϕ
4107	+	+	BBBBB	5333	+	+	EMϕ
5008	+	+	BBBBB	6009	+	+	EMϕ
6055	+	+	BBBBB	6103	+	+	EMϕ
7072	+	+	BBBBB	6118	+	+	EMϕ
0063	+	+	BBBBBB	6177	+	+	EMϕ
1256	+	+	BBBBBB	6192	+	+	EMϕ
9032	+	+	BBBBBB	7035	+	+	EMϕ
9038	+	+	BBBBBB	7079	+	+	EMϕ
5076	+	+	DDDDD	7181	+	+	EMϕ
7042	+	+	DDE	7211	+	+	EMϕ
4318	+	+	DDDDDE	7264	+	+	EMϕ
2070	+	+	DDDDDDE	7285	+	+	EMϕ
6206	+	+	DDDDDDE	7306	+	+	EMϕ; BBBBB
7175	+	+	DDDDDDE	7332	+	+	EMϕ
8078	+	+	DDDDDDE	8001	+	+	EMϕ
8416	+	+	DDDDDDE	8002	+	+	EMϕ
9060	+	+	DDDDDDE	8028	+	+	EMϕ
9514	+	+	DDDDDDE	8059	+	+	EMϕ
7030	+	+	DDDDDDDDDE	8219	+	+	EMϕ
0050	+	+	EMϕ	8261	+	+	EMϕ
0435	+	+	EMϕ	8308	+	+	EMϕ
1006	+	+	EMϕ	8414	+	+	EMϕ
1021	+	+	EMϕ	8918	+	+	EMϕ
1026	+	+	EMϕ	9012	+	+	EMϕ
1030	+	+	EMϕ	9031	+	+	EMϕ
1039	+	+	EMϕ	9061	+	+	EMϕ; BBBB
2079	+	+	EMϕ	1024	+	+	EMϕ; DDDDDDE
2088	+	+	EMϕ	1027	+	+	EMϕ; DDDDDDE
2206	+	+	EMϕ	9060	+	+	EMϕ; DDDDDDE
3062	+	+	EMϕ				

Plasmid DNA was isolated from individual transformed colonies, the presence of the predicted insert was confirmed by EcoRI digestion, and inserts were sequenced in both directions with a Big Dye kit and an ABI 3100 Prism automated sequencer. Sequences were compiled by using VECTOR NTI (InforMax). Genotypes were reported by using the convention of Allred et al. (1), in which each unique repeat type is designated by a letter, A to Z or α to ϕ. Repeat types A to E were reported by Allred et al. (1); types F to J were reported by Palmer et al. (14); types K to V were reported by de la Fuente et al. (4); type Z was reported by Futse et al. (9); and types α to ϕ were reported by Garcia-Garcia et al. (10).

A *msp1*α genotype was obtained from each of the 75 persistently infected cows (Table 1). That the genotype identified by PCR amplification and sequencing represents the true *msp1*α genotype is supported by three lines of evidence: (i) each sequence differed only in the number and sequence of repeats, with no changes in the nucleotide sequence or reading frame of the flanking 5′ and 3′ regions, which are highly conserved (1); (ii) 10 samples were reextracted, amplified, and sequenced, with the identical sequence being obtained from the independent replicates; and (iii) the size of the *msp1*α repeat region was determined by EcoRII digestion and Southern blotting, excluding possible addition or loss of repeats during PCR amplification. For verification using Southern blotting, three distinct *A. marginale* strains, with genotypes EMϕ, BBBBBB, and DDDDDDE, were isolated by inoculation of 10 ml of blood obtained from persistently infected cows (animal numbers 3201, 9038, and 8416) into each of three MSP5 CI-ELISA seronegative, *msp5* PCR negative, splenectomized calves. Each of the calves developed acute *A. marginale* bacteremia, and blood with bacteremia levels of $\geq 10^9$ organisms per ml was collected. Total DNA was PCR amplified, and the *msp1*α genotype was determined by sequencing as described above. Each of the *A. marginale* genotype sequences obtained from the inoculated calves was identical to that of the strain from persistently infected cows. For Southern blotting, nonamplified genomic DNA isolated from the blood of each calf at peak *A. marginale* bacteremia was digested with EcoRII and hybridized with a digoxigenin-labeled *msp1*α probe spanning the repeat region (14). The probe was generated by PCR using the primers 5′-CATTTCCATATACTGTGCAG and 5′-CTTGGAG CGCATCTCTCTTGCC and the PCR probe synthesis kit (Roche). The EcoRII sites in *msp1*α are external to the repeat region and thus can be used to provide an independent estimate of the number of 84- to 87-bp repeats (14). The EMϕ

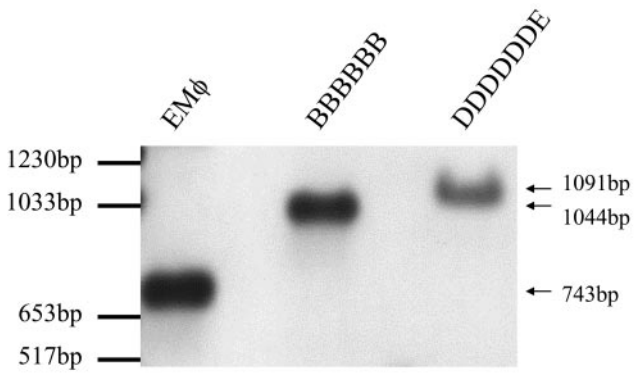

FIG. 1. Southern blot confirmation of the *msp1*α repeat structure predicted by amplicon size and sequence. DNA extracted from animals 3201 (EMϕ genotype), 9038 (BBBBBB genotype), and 8416 (DDDDDDE genotype) was EcoRII digested and Southern blotted using an *msp1*α probe. Arrows on the right designate the predicted sizes for the internal EcoRII fragments of *msp1*α for each genotype, and the positions of the molecular size markers are indicated on the left.

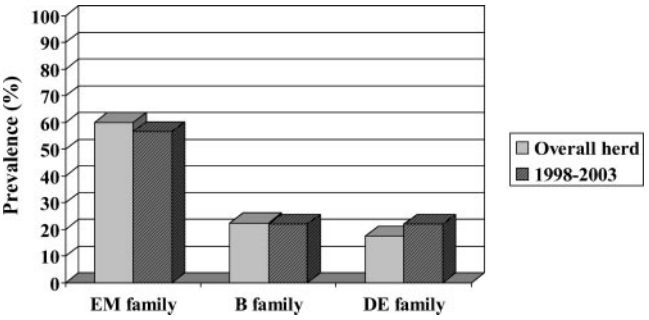

FIG. 2. *Anaplasma marginale* genotype prevalence in the herd and in animals born into and infected within the herd from 1998 to 2003.

genotype, with three repeats; the BBBBBB genotype, with six repeats; and the DDDDDDE genotype, with seven repeats, contained the predicted EcoRII repeat region fragments of 743 bp, 1,044 bp, and 1,091 bp, respectively (Fig. 1).

Eleven distinct *A. marginale msp1*α genotypes were detected from the 75 persistently infected animals (Table 1). While this is the most genotypic diversity yet reported within a single herd, it is consistent with previous studies reporting the presence and transmission of multiple distinct genotypes within herds in regions where the organism is endemic (7, 14). Interestingly, the 11 genotypes separated into three families: EMϕ, which contained a single genotype; B_x, which contained five separate genotypes with two to six B-sequence repeats; and D/E, which contained five genotypes defined by a series of two to nine D repeats with or without a terminal E repeat sequence (Table 1). The presence of closely related genotypes, exemplified by the latter two families, B_x and D/E, has previously been detected within a herd with a high prevalence of infection in eastern Oregon (14). However, that herd differed from the one in the present study in that all of the detected genotypes were closely related rather than segregated into distinct families. While it is presumed that these closely related genotypes are evolutionarily derived from one another or a common parent strain, tracking of genotypes during long-term persistent infections in the mammalian reservoir and through the cycle of tick transmission has failed to detect genotypic changes, suggesting that the rate of change and/or selection is relatively low (14).

The hypothesis that preferential strain transmission occurs would be supported by the detection of specific genotypes, or a family of genotypes, in calves born into and infected within the herd. Examination of 20 infected animals born after 1998 revealed the presence of each of the three families of genotypes, with a total of six individual genotypes. There was no statistically significant difference in genotype frequency between the herd population and the calves born into and infected within the herd (Fig. 2), as tested by using analysis of variance followed by determination of Fisher's least significant difference using a P value of ≤ 0.05 for significance. Thus, *A. marginale* genotypes in each of the three families are being maintained within the herd by ongoing transmission. The data do not support the hypothesis of preferential strain transmission and appear most consistent with transmission being stochastic relative to genotype frequency. This result may reflect equal transmission efficiency of the different strains by ticks or, alternatively, mechanical transmission that does not require *A. marginale* invasion and replication in a vector and would be predicted to reflect genotype frequency. However, an important caveat should be noted; the conclusion of stochastic transmission is limited to ongoing transmission within a herd with a high prevalence of infection, and whether this conclusion applies to disease outbreaks when widespread transmission to and within a population of naïve animals occurs is unknown. Characterization of *A. marginale* strains isolated from each of 10 sick animals during an acute outbreak revealed complete homogeneity in the msp1α genotypes (14).

Prior studies of herds naturally infected with *A. marginale* and with experimental *A. marginale* infections have indicated that individual animals are infected with only a single *msp1*α genotypically defined strain (3, 7, 14). The present study is the first to identify superinfection with two *A. marginale* strains; five animals (1024, 1027, 7306, 9060, and 9061) were each infected with two distinct genotypes. Interestingly, the two genotypes present represent different families: EMϕ and D/E were present in animals 1024, 1027, and 9060; and EMϕ and B_x were present in animals 7306 and 9061. While superinfection with *A. marginale* strains with closely related *msp1*α genotypes remains unreported, whether this reflects a true lack of occurrence or the detection of only a predominant genotype, with very low levels of a second genotype remaining undetected, is unknown. *A. marginale* superinfection is common in animals deliberately infected (live strain vaccination) with *A. marginale* subsp. *centrale*. Within a region where the organism is endemic, 64% of cattle inoculated with the live *A. marginale* subsp. *centrale* vaccine strain were subsequently infected by natural transmission of *A. marginale* and harbored both organisms (15). The basis for this marked difference between the relatively high frequency of superinfection observed following live vaccination and the low frequency in natural transmission within herds with high infection prevalence is unknown, although it may well be related to genetic distance and corresponding antigenic differences among the subspecies and strains. Resolving this question will require integrating studies of strain transmission with antigenic characterization of the

strains and the epitope specificity of the immune responses elicited during infection.

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ERRATUM

Stochastic Transmission of Multiple Genotypically Distinct *Anaplasma marginale* Strains in a Herd with High Prevalence of *Anaplasma* Infection

Guy H. Palmer, Donald P. Knowles, Jr., Jose-Luis Rodriguez, David P. Gnad, Larry C. Hollis, Twig Marston, and Kelly A. Brayton

Program in Vector-borne Diseases, Washington State University, and Animal Diseases Research Unit, U.S. Department of Agriculture-Agricultural Research Service, Pullman, Washington, and Department of Clinical Sciences and Department of Animal Sciences and Industry, Kansas State University, Manhattan, Kansas

Volume 42, no. 11, p. 5381–5384, 2004. Page 5382, Table 1: The *msp1*α genotype for animal 6192 should read "BBBBB."